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Escherichia coli O157:H7 Colonization in Cattle following Systemic and Mucosal Immunization with Purified H7 Flagellin▽

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Escherichia coli* O157:H7 is an important pathogen of humans. Cattle are most frequently identified as the primary source of infection, and therefore, reduction in *E. coli* O157:H7 prevalence in cattle by vaccination represents an attractive strategy for reducing the incidence of human disease. H7 flagella have been implicated in intestinal-epithelial colonization of *E. coli* O157:H7 and may represent a useful target for vaccination. In this study, calves were immunized either systemically with H7 flagellin by intramuscular injection or mucosally via the rectum with either H7 or H7 incorporated into poly(DL-lactide-co-glycolide) microparticles (PLG:H7). Systemic immunization resulted in high levels of flagellin-specific immunoglobulin G (IgG) and IgA in both serum and nasal secretions and detectable levels of both antibody isotypes in rectal secretions. Rectal administration of flagellin resulted in levels of rectal IgA similar to those by the intramuscular route but failed to induce any other antibody response, whereas rectal immunization with PLG:H7 failed to induce any H7-specific antibodies. Following subsequent oral challenge with *E. coli* O157:H7, reduced colonization rates and delayed peak bacterial shedding were observed in the intramuscularly immunized group compared to nonvaccinated calves, but no reduction in total bacterial shedding occurred. Rectal immunization with either H7 or PLG:H7 had no effect on subsequent bacterial colonization or shedding. Furthermore, purified H7-specific IgA and IgG from intramuscularly immunized calves were shown to reduce intestinal-epithelial binding in vitro. These results indicate that H7 flagellin may be a useful component in a systemic vaccine to reduce *E. coli* O157:H7 colonization in cattle.

Enterohemorrhagic *Escherichia coli* (EHEC) is a zoonotic pathogen of worldwide importance, causing severe diarrhea (hemorrhagic colitis) and, in a small percentage of cases, hemolytic-uremic syndrome in humans. Ruminants are an important reservoir of EHEC, and human infections are frequently associated with direct or indirect contact with ruminant feces, particularly those derived from cattle (16, 26, 34, 36). Coincidentally, strategies to reduce the carriage of EHEC in ruminants are predicted to lower the incidence of human disease (reviewed in reference 36), and stochastic simulation models predict that cattle are a key control point to reduce EHEC infections in humans (22).

An attractive strategy to reduce EHEC colonization in cattle is by vaccination. A number of EHEC vaccines have been evaluated in cattle and have primarily focused on immunization with bacterial proteins encoded by genes located within the locus of enterocyte effacement (LEE) that are known to play key roles in EHEC colonization of the bovine intestine (7, 13, 32, 41). These include immunization with recombinant EspA (14), recombinant intimin (40), and a secreted protein preparation containing Tir and proteins of the type III secretion system (35). In addition, immunization with recombinant EHEC factor for adherence (encoded by *efa-1*), another protein implicated in bovine intestinal colonization (37), and formalin-inactivated EHEC bacterin have been evaluated (40). However, with the exception of immunization with the secreted protein preparation, which resulted in a reduction in bacterial fecal shedding in calves following experimental challenge and reduced prevalence of EHEC in a small- but not a large-scale field trial (39), all EHEC vaccines evaluated have had no effect on subsequent EHEC colonization in cattle.

The reasons for the lack of success of EHEC vaccines to date could be twofold. First, it may indicate that additional vaccine targets are required. Second, as EHEC is nonpathogenic in cattle and is restricted to the intestinal epithelium and gut lumen (reviewed in reference 36), intestinal mucosal antibodies to bacterial epithelial adhesins are likely to represent key effectors for reduction of intestinal colonization in cattle. Indeed, passive immunization of piglets with colostrum containing intimin-specific antibodies has been shown to confer resistance to subsequent EHEC challenge, supporting a role for mucosal antibodies in reducing bacterial colonization in vivo

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(10). However, in previous EHEC vaccine trials, antigen-specific mucosal antibody responses postimmunization either were not measured (35) or were poor (14, 40). Therefore, the lack of vaccine efficacy may in part be due to poor levels of antigen-specific antibody at intestinal mucosal surfaces.

In North America and Europe, the predominant EHEC serotype affecting humans is O157:H7 (1, 2). The principal site of *E. coli* O157:H7 colonization in cattle is the terminal rectum (31). Work in our laboratory has indicated that H7 flagella play an important role in initial binding of *E. coli* O157:H7 to bovine primary rectal epithelial cells in vitro (27), and Erdem et al. have also recently demonstrated that the presence of H7 flagella is important in bacterial adherence to bovine intestinal-tissue explants (15). Furthermore, H7 flagella have been shown to play a role in *E. coli* O157:H7 colonization of chickens in vivo (3), and flagella of a number of other bacteria, including *Clostridium difficile* (38), *Pseudomonas aeruginosa* (25), and enteropathogenic *E. coli* (17), have been demonstrated to act as epithelial adhesins. Together, these observations suggest that H7 flagella may represent an additional target for *E. coli* O157:H7 vaccination in cattle.

In this study, we evaluated the effects of systemic and mucosal immunization with purified H7 flagellin, the main structural component of H7 flagella, on subsequent *E. coli* O157:H7 colonization in cattle. In an attempt to induce high levels of mucosal antibodies at the principle site of colonization of *E. coli* O157:H7 in cattle, i.e., the terminal rectum, mucosal immunizations with either H7 flagellin alone or H7 incorporated into poly(DL-lactide-co-glycolide) (PLG) microparticles (PLG:H7) were administered onto the rectal mucosa, which in cattle possesses characteristics of an immune inductive site (28). Furthermore, we performed a detailed analysis of H7-specific mucosal antibody levels following immunizations using previously validated mucosal sampling protocols (29).

MATERIALS AND METHODS

Purification of *E. coli* O157:H7 flagellin and encapsulation into PLG microparticles. Flagellin (H7) was isolated from *E. coli* O157:H7 (*stx* mutant) strain ZAP984, a *LEE4* deletion mutant derived from strain ZAP198 (32) by acid dissociation, neutral-pH reassociation, and ammonium sulfate precipitation as previously described (19). This protocol results in the purification of flagellin monomers, which spontaneously repolymerize into flagellar filaments at neutral pH. Purity was verified using polyacrylamide gel electrophoresis, followed by Simply Blue staining (Invitrogen, San Diego, CA) and by Western blotting.

PLG:H7 microparticles were prepared using the water/oil/water solvent evaporation technique as previously described (20). Briefly, 100 μl of H7 flagellin at 10 mg/ml in distilled water was emulsified with 2 ml of a 5% solution of PLG (Sigma-Aldrich, St. Louis, MO) in dichloromethane at 16,000 rpm for 2 min using a T25 Basic Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany) to produce a water-in-oil emulsion. The emulsion was subsequently homogenized with 20 ml of 5% polyvinyl alcohol solution at 19,000 rpm for 2 min to produce a stable water/oil/water emulsion. The secondary emulsion was stirred for 12 to 18 h at room temperature (RT) and pressure to allow solvent evaporation to occur and subsequent microparticle preparation. The microparticles were collected by centrifugation, washed three times with distilled water, and lyophilized. This procedure was repeated on five separate occasions, and the resultant microparticles were pooled for subsequent immunizations.

The protein content and ratio of surface-bound to encapsulated protein of each batch of microparticles was assessed using a bicinchoninic protein assay kit (Pierce, Rockford, IL) following removal of surface protein from a known weight of microparticles with 2% sodium dodecyl sulfate and subsequent dissolution of the particles in 0.5 M NaOH to release encapsulated protein. The released protein was subjected to Western blotting. The particle size and the number of particles per mg PLG:H7 was estimated using a Z1 Coulter particle counter (Beckman Coulter Inc., Fullerton, CA).

Western blotting. Samples of H7 flagellin or digested PLG:H7 microspheres were separated by polyacrylamide gel electrophoresis using NuPAGE 4 onto 12% Bis-Tris gels (Invitrogen) under reducing conditions and transferred to nitrocellulose membranes (pore size, 0.2 μm) using the Xcell II blotting system (Invitrogen) according to the manufacturer's instructions. The membranes were subsequently washed in phosphate-buffered saline (PBS) containing 0.5 M NaCl and 0.5% (vol/vol) Tween 80 for 1 h at RT. The blots were incubated for 1 h at RT with rabbit polyclonal anti-H7 antibody (Mast-Assure, Bootle, United Kingdom) diluted 1:500 in PBS containing 0.5 M NaCl and 0.5% (vol/vol) Tween 80, followed by incubation for 1 h at RT with a 1:2,000 dilution of goat anti-rabbit polyclonal antibody conjugated to horseradish peroxidase (HRP) (Dakocytomation, Ely, United Kingdom). Controls included omission of the primary antibody and replacement of primary antibody with normal rabbit serum. HRP was detected after the final washing by incubating the blots with ECL Plus reagent (GE Healthcare, Little Chalfont, United Kingdom).

Immunization protocol and oral bacterial challenge. Immunizations and oral bacterial challenges were performed at the Moredun Research Institute under Home Office license 60/3179. Ethical approval was obtained from the Moredun Research Institute Animal Experiments Committee. Four groups of eight conventionally reared male Holstein-Friesian calves were immunized on three separate occasions at 2-week intervals as follows: group 1 received 60 μg H7 flagellin plus 5 mg Quil A (Brenntag Biosector, Frederikssund, Denmark) in 2 ml PBS intramuscularly (i.m.); group 2 received 60 μg H7 flagellin in 1 ml PBS administered intrarectally using a pastette inserted approximately 4 cm beyond the anus; group 3 received 60 μg H7 flagellin incorporated into PLG microparticles and resuspended in 1 ml PBS intrarectally; group 4 received no immunizations (nonvaccinated control group). The average age of the calves at the time of the first immunization was 9 ± 2 weeks, and fecal samples obtained from each calf prior to immunization were confirmed to be negative for *E. coli* O157:H7 by immunomagnetic separation performed according to the manufacturer's instructions (Dynabeads anti-*E. coli* O157; Invitrogen).

Ten days after the last immunization, the calves were orally challenged with 10^{10} CFU naldixic acid-resistant *E. coli* O157:H7 (*stx* mutant) strain ZAP198, and viable *E. coli* O157:H7 bacteria per gram of surface feces were enumerated once daily for 3 weeks postchallenge by plating them onto sorbitol MacConkey agar plates containing 15 μg/ml naldixic acid (Oxoid, Basingstoke, United Kingdom) as previously described (31). To estimate the total bacterial fecal shedding, daily bacterial counts were plotted versus time for each calf, and the area under the shedding curve (AUC) was calculated. In addition, bacterial uptake rates (as defined by an increasing fecal bacterial count from 3 days postchallenge) were recorded. Serum, nasal secretions, and rectal swabs were collected as described previously (29) 4 days prior to the first immunization, 1 week after each immunization, and 2 weeks after oral bacterial challenge. The calves were euthanized 3 weeks after bacterial challenge, and abomasal and small-intestinal swabs were collected, in addition to serum, nasal secretions, and rectal swabs.

Quantification of anti-H7 flagellin and anti-O157 LPS antibodies. H7 flagellin-specific immunoglobulin A (IgA) and IgG antibodies were quantified in serum, nasal secretions, and intestinal-swab samples by indirect enzyme-linked immunosorbent assay (ELISA) as described previously (29). To measure anti-O157 lipopolysaccharide (LPS) antibodies, plates were coated with 0.1 μg/well O157 LPS conjugated to polymixin as previously described (9) and subsequently processed identically to the H7 ELISAs. Optimum sample dilutions were determined following serial dilution of representatives from each to ensure that the color reaction product at an optical density at 492 nm (OD_{492}) for the samples was on the linear part of the curve. Samples were diluted 1:1,000, 1:10, and 1:2.5 for serum, nasal secretions, and intestinal-swab samples, respectively, for IgG ELISAs and 1:10, 1:100, and 1:2.5 for serum, nasal secretions, and intestinal-swab samples, respectively, for IgA ELISAs. ODs obtained for intestinal-swab samples were normalized to the total IgA, measured using a sandwich ELISA obtained from Bethyl Laboratories Inc. (Montgomery, TX), and interplate variation was normalized to a positive control. Western blotting was also performed using ECL Plus reagent (GE Healthcare) on selected preimmunization, postimmunization, or post-bacterial-challenge serum, nasal secretions, and rectal-swab samples from each immunization group to confirm the specificity of the antibody responses measured by ELISA.

Purification of anti-H7 flagellin IgA and IgG. Neat pre- and postimmunization nasal-secretion samples from five i.m. H7-immunized calves were clarified by centrifugation at 11,000 × *g* for 5 min. Protein fractionation of 0.5 ml nasal secretion was carried out by gel filtration chromatography using a Superdex 200 10/300 column and the AKTA fast-protein liquid chromatography system (GE Healthcare Biosciences AB, Uppsala, Sweden). The column was equilibrated

with PBS containing 0.04% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} (Sigma-Aldrich), and 1-ml fractions were collected. Total IgA and IgG were quantified in fractions 7 to 16 using commercial sandwich ELISAs (Bethyl Laboratories Inc.). Undiluted fractions were also subjected to H7-specific IgA and IgG ELISAs. In addition, H7-specific IgM antibodies were measured by ELISA using anti-bovine IgM conjugated to HRP (AbD Serotec, Kidlington, United Kingdom).

In vitro intestinal epithelial binding assay. Stationary-phase minimal essential medium-HEPES cultures of *E. coli* O157:H7 strain NCTC12900 (which has an H7 flagellin sequence identical to that of strain ZAP198 used in oral bacterial challenges) or an isogenic aflagellar mutant strain (3) (both obtained from M. J. Woodward and R. LaRagione, VLA Weybridge, United Kingdom) were diluted 1:10 in minimal essential medium-HEPES and incubated at 37°C with shaking (200 rpm) to an OD_{600} of 0.3 to 0.4. Two hundred-microliter aliquots of bacterial culture were subsequently incubated for 30 min at RT with 200 μl of either anti-H7 IgA or anti-H7 IgG purified from the nasal secretions of i.m. H7-immunized calves. Both the IgA and IgG fractions had been previously diluted to give an OD_{492} of 1.0 on anti-H7 IgA and IgG ELISA, respectively. Bacteria were also incubated with an equivalent concentration of total IgA and IgG purified from the preimmunization nasal secretions of the same calves. Following antibody incubation, confluent bovine primary rectal epithelial cells prepared as described previously (12) were separately infected in duplicate with 100 μl/well of each bacterial suspension and incubated for 1 h at 37°C in a humidified 5% CO_2 atmosphere. Nonadherent bacteria were removed by washing them three times in PBS, and adherent viable bacteria were enumerated following dispersion with 1% PBS-0.1% (vol/vol) Triton X-100, serial 10-fold dilution, and plating onto LB agar plates. To account for potential differences in the numbers of bacteria inoculated into each well, the number of bacteria within each sample following the antibody incubation step was also determined, and the data were used to normalize the resultant adherent bacterial counts. The assay was repeated with pre- and postimmunization samples derived from five separate calves.

Statistical analyses. All statistical analyses were performed using the statistical package Genstat, 7th edition. ELISA and bacterial-shedding data were $\log_{10}$ transformed before analysis to ensure that observations within each group had an approximately normal distribution with a common variance. ELISA data were analyzed in two ways: first, data from the different immunization groups 1 week after the final immunization and at postmortem (for abomasal and small-intestinal swabs only) were compared using one-way analysis of variance (ANOVA); secondly, differences between postimmunization and post-bacterial-challenge ELISA data within each immunization group were compared using a paired *t* test. A repeated-measures model was fitted to the fecal bacterial shedding data with the lack of independence between successive measurements on the same individual modeled with an autoregressive correlation structure. The parameters of the models were estimated using the REML directive in Genstat. AUC data from different immunization groups were compared using a Kruskal-Wallis test, and colonization rates between groups were compared using a Fisher's exact test. Bacterial binding data were compared using one-way ANOVA. All ANOVA was followed by the Tukey post hoc test for pairwise comparison of means. Correlations between normally distributed data were performed using Pearson's correlation. *P* values of <0.05 were considered significant.

RESULTS

Characterization of PLG:H7 microparticles. Approximately 94% of the microparticles were <3 μm in diameter, with a further 5% of the particles between 3 and 5 μm in diameter. The efficiency of encapsulation of H7 flagellin into microparticles ranged between 35 and 40%. Of the total encapsulated protein, it was estimated that 24 to 35% was surface bound, with the remaining 65 to 76% internalized into the microparticles. The number of particles per mg of PLG:H7 of the pooled microparticles from all batches was estimated to be approximately 8×10^6 particles/mg PLG:H7, and each mg of PLG:H7 was equivalent to 6 μg H7. Digestion of microparticles released a single protein of approximately 60 kDa (Fig. 1A), which reacted strongly with commercial anti-H7 antibody following Western blotting (Fig. 1B). A band of approximately 120 kDa was also weakly reactive to commercial anti-H7 antibody. However, this band is commonly seen in our H7 flagellar preparations and has been previously identified as H7 flagellin by matrix-assisted laser desorption ionization–time of flight mass spectrometry (27), suggesting that the band may represent H7 with posttranslational modifications or H7 dimers.

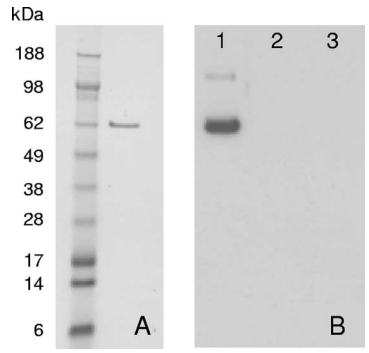

FIG. 1. Evaluation of PLG:H7 microparticles by polyacrylamide gel electrophoresis and Western blot analysis. (A) Coomassie blue-stained gel of protein released from PLG microparticles following dissolution with sodium dodecyl sulfate and NaOH. (B) Western blot of protein released from PLG microparticles with rabbit polyclonal anti-H7 antibody (lane 1), normal rabbit serum (lane 2), or no primary antibody (lane 3).

H7-specific antibody responses following immunization with H7 flagellin and subsequent oral bacterial challenge. Serum, nasal, and rectal H7-specific antibody responses are shown in Fig. 2. High titers of anti-H7 IgG and IgA were induced in both serum and nasal secretions following i.m. injection of H7, and IgA antibody levels reached peak levels after the second immunization, whereas IgG antibody levels continued to rise (Fig. 2A to D). In contrast, per-rectal immunization with either H7 or PLG:H7 failed to induce any significant serum or nasal H7-specific antibody response. Subsequent oral challenge with *E. coli* O157:H7 resulted in a small but significant increase in serum anti-H7 IgG within all immunized and control groups compared to postimmunization levels ($P < 0.01$ for all groups) (Fig. 2A). Bacterial challenge also resulted in a small but significant increase in nasal anti-H7 IgG ($P < 0.05$) (Fig. 2C) and a significant increase in nasal and serum IgA in animals from rectally immunized and control groups, but not from i.m. immunized calves ($P < 0.01$ for both) (Fig. 2B and D).

Anti-H7 IgG antibodies were detected in rectal-swab samples following i.m. immunization with H7, but not with any other immunization group (Fig. 2E). Rectal H7-specific IgA antibodies were induced following both i.m. vaccination and rectal immunization with H7 in PBS, and 1 week after the final immunization (day 42), the levels of anti-H7 IgA were similar in the two groups (Fig. 2F). In contrast, rectal immunization with PLG:H7 failed to induce any rectal anti-H7 IgA antibodies. Following bacterial challenge, a significant increase in rectal anti-H7 IgA, but not IgG, was observed in both rectally immunized groups and the nonvaccinated control group ($P < 0.01$ for all groups). No significant increase in H7-specific IgA or IgG was observed in i.m. vaccinated calves in response to bacterial challenge.

Postmortem abomasal and small-intestinal H7-specific antibody levels are shown in Fig. 3. Significantly higher levels of anti-H7 IgG antibodies were found in the abomasum and small

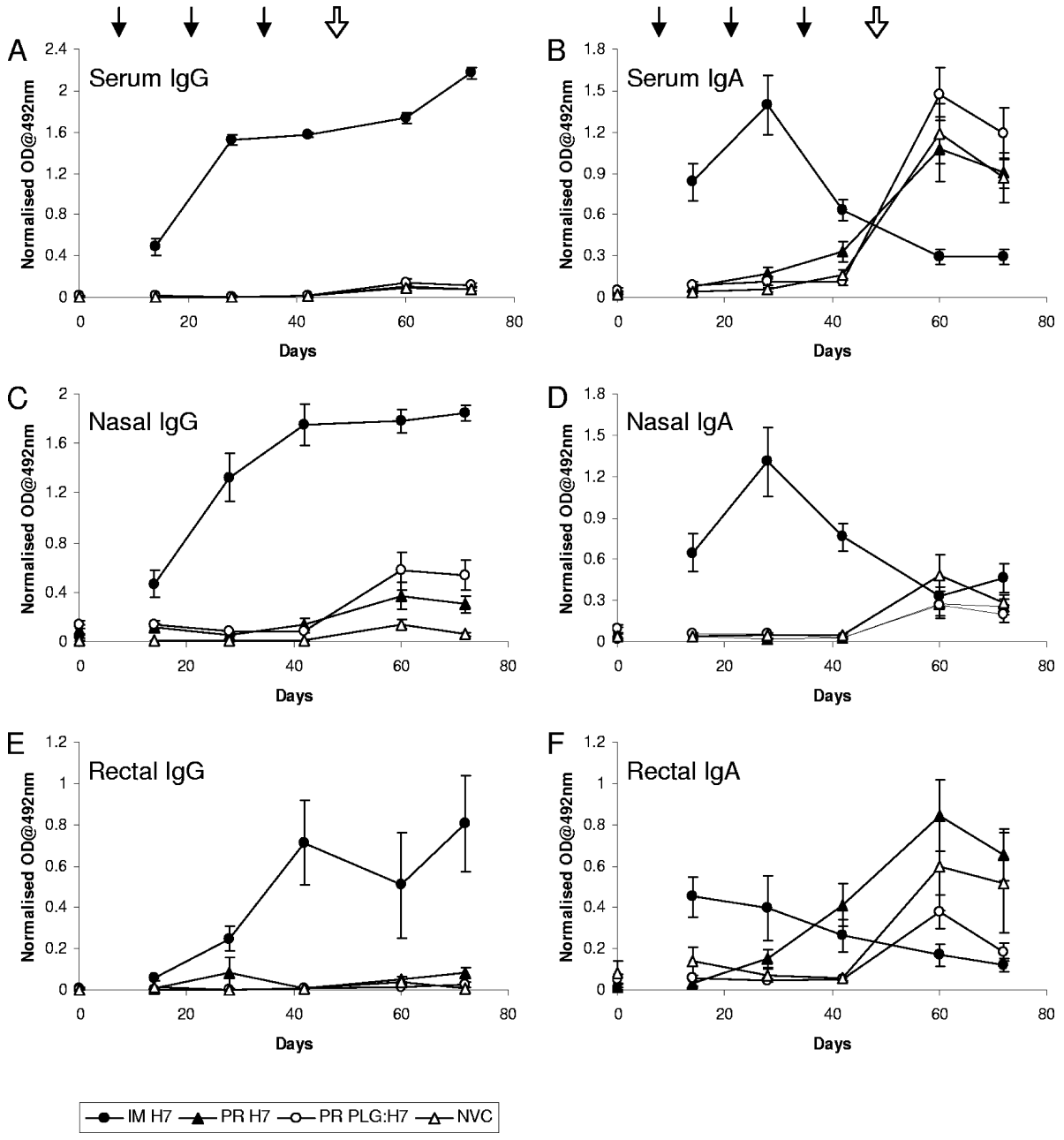

FIG. 2. Levels of H7-specific IgG and IgA measured by ELISA in serum (A and B), nasal secretions (C and D), and rectal-swab samples (E and F) from calves following either systemic or mucosal immunization with H7 flagellin. The values represent the mean value ± the standard error of the mean for eight calves at each time point. The solid arrows indicate the timing of immunizations; the open arrow indicates the timing of subsequent oral bacterial challenge with *E. coli* O157:H7. IM H7, i.m. H7; PR H7, rectal immunization with H7 in PBS; PR PLG:H7, rectal immunization with H7 encapsulated in PLG microparticles; NVC, nonvaccinated controls.

intestines of i.m. immunized calves compared to rectally immunized and nonvaccinated calves (one-way ANOVA; main P values for both < 0.0001; significance at the 1% level using Tukey's pairwise comparisons). No differences in abomasal or small-intestinal H7-specific IgA levels were observed between immunization groups.

Western blotting was performed on selected preimmunization, postimmunization, or post-bacterial-challenge samples from each immunization group. This confirmed that immunoreactivity was primarily directed against H7 flagellin (data not shown).

Correlation between H7- and LPS-specific antibody responses following oral bacterial challenge. Oral bacterial challenge with *E. coli* O157:H7 resulted in an increase in H7-specific IgA antibodies in mucosal and serum samples from all groups, with the exception of the i.m. immunized group. To determine whether H7 responses following bacterial challenge in the i.m. immunized group were correlated with levels of

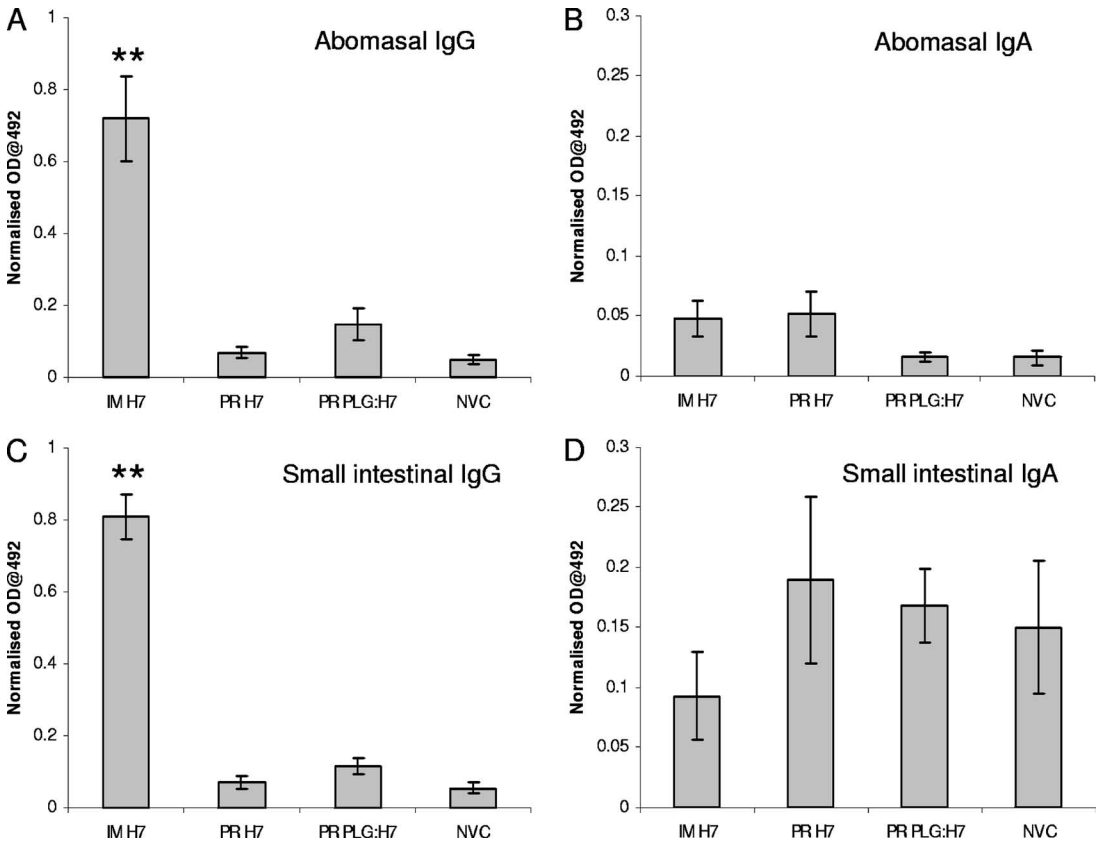

FIG. 3. Levels of H7-specific IgG and IgA measured by ELISA in abomasal (A and B) and small-intestinal (C and D) swabs of calves at postmortem following either systemic or mucosal immunization with H7 and subsequent oral challenge with *E. coli* O157:H7. The values represent the mean value ± standard error of the mean for eight calves at each time-point. **, significantly different from the other three groups by ANOVA (main P value < 0.0001; significance at the 1% level using Tukey's pairwise comparisons). IM H7, i.m. H7; PR H7, rectal immunization with H7 in PBS; PR PLG:H7, rectal immunization with H7 encapsulated in PLG microparticles; NVC, nonvaccinated controls.

bacterial colonization, anti-O157 LPS IgA antibodies (used as an indicator of *E. coli* O157:H7 mucosal immune responses) (30) were measured in serum, nasal secretions, and rectal-swab samples in i.m. immunized and nonvaccinated calves 12 days post-bacterial challenge. Correlations between anti-H7 and LPS IgA responses at day 12 postchallenge are shown in Table 1. In nonvaccinated calves, there was a significant positive correlation between anti-H7 and LPS antibody responses in serum, nasal secretions, and rectal-swab samples. In i.m. immunized calves, a significant positive correlation existed between H7 and LPS antibody levels in rectal swabs, but not in serum or nasal secretions.

TABLE 1. Correlation coefficients between anti-H7 and anti-O157 LPS IgA antibodies following oral challenge with *E. coli* O157:H7

Sample	i.m. H7-immunized group		Nonvaccinated group	
	r^a	P value	r^a	P value
Serum	0.456	0.303	0.900	0.002
Nasal secretions	0.524	0.182	0.861	0.006
Rectal swabs	0.865	0.006	0.879	0.004

[a] Pearson's correlation coefficient calculated using log-transformed data.

***E. coli* O157:H7 colonization following immunization with H7 flagellin.** Following immunization, oral bacterial challenge with *E. coli* O157:H7 resulted in successful colonization of three of eight calves in the i.m. immunized group compared to 100% of rectally immunized and six of eight nonvaccinated calves. Comparison of uptake rates between groups using Fisher's exact test identified significant differences in uptake rates between the groups ($P = 0.006$), with the i.m. immunized group having a markedly lower uptake rate than the other groups.

Daily mean bacterial counts for colonized calves only are shown in Fig. 4A. There was an overall immunization times time interaction ($P = 0.009$) that was reflected in higher mean bacterial counts in the i.m. immunized group compared to rectally immunized and control groups on days 12 and 13. No differences in mean bacterial counts were identified between groups at other time points. Peak bacterial shedding occurred between days 10 and 14 in colonized calves from the i.m. immunized group, whereas peak shedding in colonized calves from rectally immunized and control groups occurred between days 5 and 8. To estimate total bacterial shedding, AUCs were calculated for each calf and are shown in Fig. 4B. No significant differences in AUCs were identified between immunization and control groups,

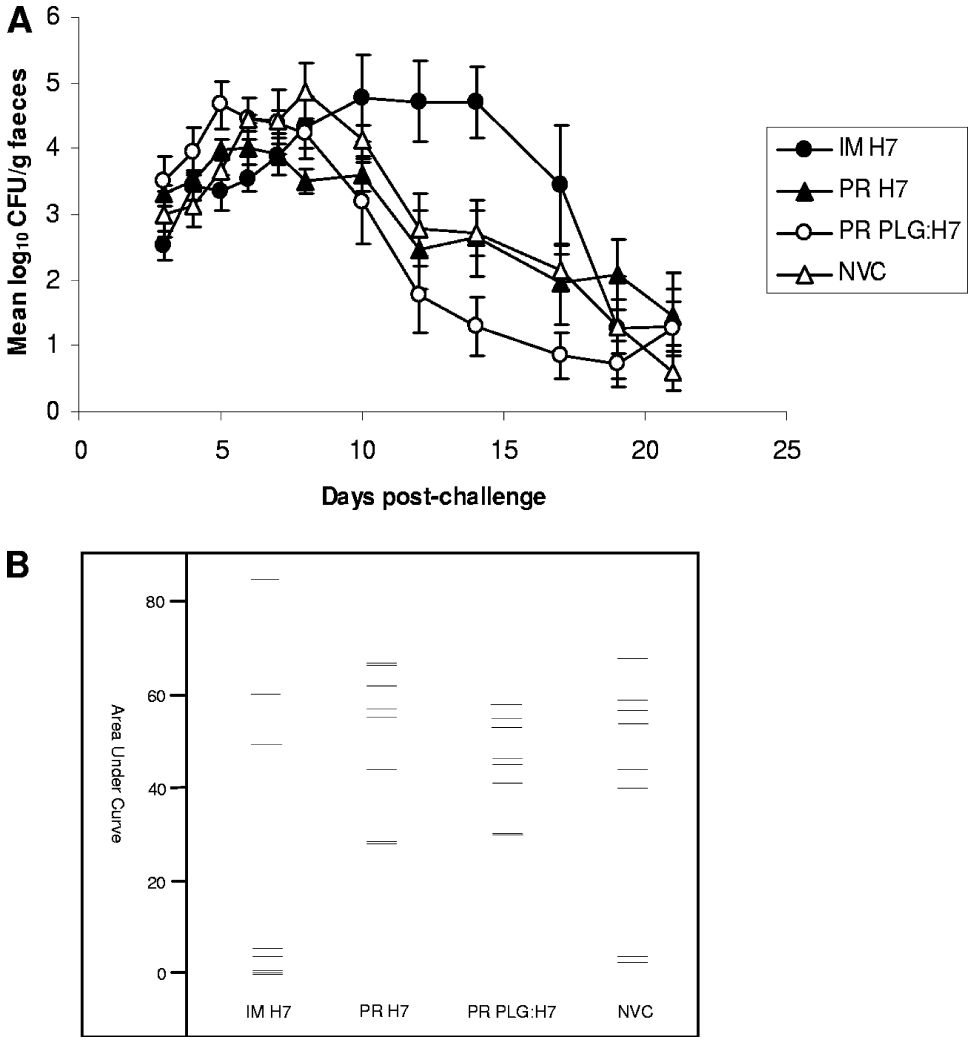

FIG. 4. Fecal shedding of *E. coli* O157:H7 following oral challenge of calves previously vaccinated either systemically or mucosally with H7 flagellin. The calves were immunized on three separate occasions and challenged 10 days after the final immunization. (A) Mean daily fecal count ± standard error of the mean for colonized calves only. (B) Rug plot of AUC data calculated over the total 21-day shedding period for each calf. Each horizontal line indicates an individual value for each calf. IM H7, i.m. H7; PR H7, rectal immunization with H7 in PBS; PR PLG:H7, rectal immunization with H7 encapsulated in PLG microparticles; NVC, nonvaccinated controls.

indicating no differences in total bacterial shedding between these groups.

Effects of anti-H7 IgA and IgG on intestinal epithelial binding of *E. coli* O157:H7 in vitro. To determine if anti-H7 IgG and/or IgA isotypes are effective at inhibiting *E. coli* O157:H7 intestinal epithelial binding, wild-type *E. coli* O157:H7 or an isogenic aflagellar mutant strain was incubated with either anti-H7 IgG or IgA purified by gel filtration chromatography from nasal secretions derived from five i.m. immunized calves, and subsequent bacterial binding to bovine primary rectal epithelial cells was evaluated. Gel filtration fractions were analyzed for total IgA/IgG and H7-specific IgA/IgG. A representative graph of total IgA and IgG levels in fractions obtained from one calf is shown in Fig. 5A, and a graph of anti-H7 antibody levels in fractions from pre- and postimmunization nasal secretions from the same calf is shown in Fig. 5B. IgA was present in fractions 7 to 11 from all calves, with maximal total IgA present in fraction 9. The peak total IgG concentration was present in fraction 12, with small quantities of IgG present in fractions 7 to 9. Measurement of anti-H7 IgA and IgG antibodies in postimmunization fractions revealed maximal anti-H7 IgA and IgG antibodies in fractions 9 and 12, respectively. No anti-H7 IgA was detected in IgG-enriched fractions. However, some anti-H7 IgG activity was detected in IgA-enriched fractions. Minimal reactivity to anti-H7 IgA and IgG ELISAs was observed in fractions obtained from preimmune nasal secretions from the same calves. Anti-H7 IgM antibodies were also measured in all fractions and were not detected (data not shown).

The effects of anti-H7 IgA- or IgG-enriched fractions (9 and 12, respectively) on epithelial binding are shown in Fig. 5C and D. Incubation of wild-type *E. coli* O157:H7 with both anti-H7 IgA and IgG fractions resulted in a significant reduction in bacterial binding to bovine rectal epithelial cells compared to incubation with preimmune IgA and IgG fractions (Fig. 5C), with both isotypes inhibiting bacterial binding to similar de-

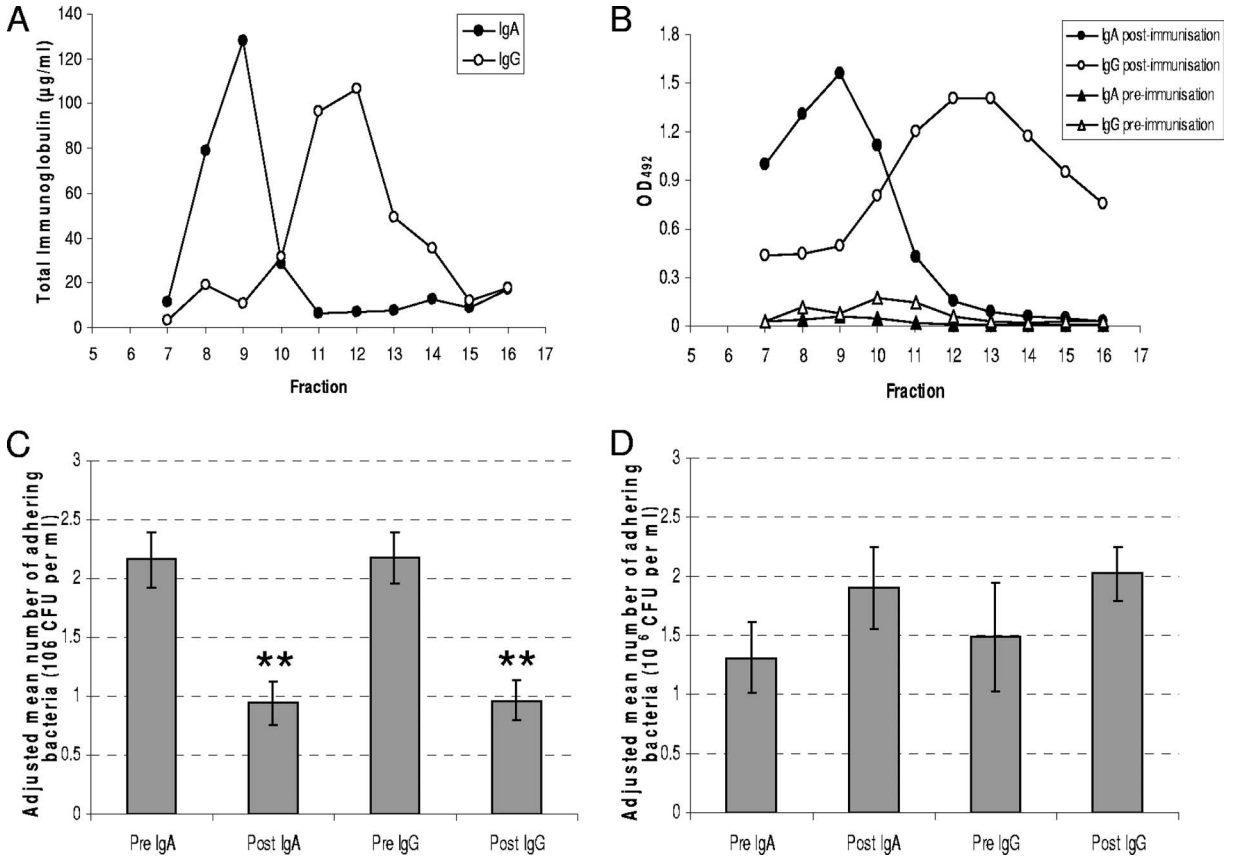

FIG. 5. (A) Total IgA and IgG levels in gel filtration fractions of nasal secretions from one calf immunized with H7 flagellin by the i.m. route. (B) Levels of H7-specific IgA and IgG in gel filtration fractions determined by ELISA from one calf before and after i.m. immunization with H7 flagellin. (C) Adherence of *E. coli* O157:H7 strain NCTC12900 to bovine primary rectal epithelial cells after incubation with IgA- or IgG-rich gel filtration fractions (9 and 12, respectively) derived from nasal-secretion samples from calves pre- and post-i.m. immunization with H7 flagellin. (D) Adherence of an isogenic aflagellar mutant strain of *E. coli* O157:H7 strain NCTC12900 to bovine primary rectal epithelial cells after incubation with IgA- or IgG-rich gel filtration fractions derived from nasal-secretion samples from calves pre- and post-i.m. immunization with H7 flagellin. The values represent the mean number of adhering bacteria ± standard error of the mean following incubation with samples obtained from five calves. **, significantly different from counts obtained following incubation with preimmune fractions by ANOVA (main P value $<$ 0.0001; significance at the 1% level using Tukey's pairwise comparisons).

grees (one-way ANOVA; main P value $<$ 0.0001; significance at the 1% level using Tukey's pairwise comparisons). No significant differences in bacterial binding were observed following incubation of the isogenic aflagellar mutant strain with either antibody isotype (Fig. 5D), indicating that the observed inhibition of bacterial binding was a flagellin-specific effect.

DISCUSSION

Systemic immunization with H7 flagellin resulted in reduced colonization rates and delayed peak bacterial shedding in colonized calves by approximately 1 week. However, no effect on total bacterial shedding following systemic immunization was observed. In contrast, rectal immunization with either H7 alone or H7 incorporated into PLG microparticles had no effect on subsequent bacterial colonization or shedding. Alterations in bacterial shedding following systemic H7 immunization correlated with a widespread mucosal antibody response to H7 consisting of both IgG and IgA isotypes: anti-H7 IgG was detectable postmortem throughout the gastrointestinal tract, and anti-H7 IgA was detectable in both rectal-swab samples and nasal secretions, suggesting that i.m. immunization resulted in induction of an IgA response at multiple mucosal sites. The lack of any effect on bacterial colonization following rectal immunization with H7 in PBS occurred despite induction of a local (rectal) IgA response. Immunization with PLG:H7 failed to induce any detectable anti-H7 antibodies, possibly due to either insufficient uptake of antigen at the rectal mucosa or alterations of the flagellin epitopes during the encapsulation process. However, the former is more likely than the latter, as Western blotting of flagellin released from the microparticles demonstrated that the released flagellin retained at least a proportion of its B-cell epitopes.

Western blotting demonstrated that the majority of antibody responses induced following vaccination were directed against H7 flagellin. This suggests that the slight protective responses observed in this study were primarily due to immune responses against the flagellin protein. However, we cannot rule out the possibility that other minor contaminants within the vaccine preparation, such as LPS, may have contributed to the altered bacterial shedding observed in i.m. immunized calves. Never-

theless, by purifying flagellin from a *stx* mutant *LEE4* deletion mutant strain of *E. coli* O157:H7, we could at least be confident that our vaccines were not contaminated with proteins encoded by the *LEE4* operon, known to be required for epithelial colonization (32), or Shiga toxin.

Although different strains of *E. coli* O157:H7 were used for in vitro binding assays and oral challenges, which may differ slightly in their epithelial binding dynamics, both strains shared identical H7 flagellin sequences. Therefore, the observation that both IgA and IgG isotypes were effective at inhibiting bacterial binding in vitro in an H7-specific manner suggests that these anti-H7 antibodies may have been similarly effective at inhibiting bacterial epithelial binding in vivo. As both IgA and IgG isotypes are key immunoglobulins present in bovine intestinal secretions (6, 33), this raises two possible explanations for the altered bacterial shedding observed following i.m. immunization but not rectal immunization with H7. First, anti-H7 antibodies may be required throughout the whole intestinal tract and not just at the rectum to have an effect on subsequent *E. coli* O157:H7 colonization in vivo. This appears likely, as, although *E. coli* O157:H7 preferentially colonizes at the terminal rectal mucosa, the bacterium also colonizes at other intestinal sites, including the forestomachs, abomasum, duodenum, ileum, cecum, and colon (4, 8, 31). Secondly, as combined levels of H7-specific IgG and IgA antibodies were higher at the rectal mucosa following i.m. immunization than following rectal H7 immunization, it may be that the overall quantity of anti-H7 antibodies at the terminal rectum is crucial for altering bacterial colonization in vivo. In either case, the effect of i.m. immunization with H7 appeared to be restricted to either a reduction in initial bacterial uptake or a delay in peak colonization but had no effect on the ability of the bacteria to reach high levels in colonized calves.

It was shown in this study that anti-H7 antibodies resulted in around a 50% reduction, but not complete elimination, of bacterial epithelial binding in vitro. This suggests that *E. coli* O157:H7 possesses additional mechanisms for intestinal epithelial attachment beyond H7-flagellin-mediated processes. Indeed, a recent study in cattle has demonstrated that knocking out the gene encoding H7 flagellin (*fliC*) had no significant effect on subsequent colonization of cattle with *E. coli* O157, indicating that intestinal epithelial binding in vivo can occur independently of H7 (11). Therefore, the results from this study indicate that although H7 flagellin may be a useful component in a systemic vaccine against *E. coli* O157:H7 in cattle, additional bacterial antigens would need to be included to significantly reduce total bacterial fecal shedding.

An interesting finding in this study is that an H7-specific mucosal IgA response was induced in both nasal secretions and rectal-swab samples following i.m. immunization with H7. This finding is unusual, as systemic routes of immunization generally result in poor mucosal IgA levels (23, 24). However, confidence in the result can be gained by the following observations: first, previous analyses of the mucosal sampling techniques employed in this study have shown that the majority of IgA present in the mucosal samples is locally, i.e., mucosally derived (29). Secondly, analysis of gel filtration fractions of nasal secretions from i.m. immunized calves indicate that the anti-bovine IgA antibodies employed in the H7 ELISA do not appear to cross-react with bovine IgG to any great extent.

The underlying mechanisms of mucosal response generation following systemic H7 immunization are unclear. It may be that the calves in this study were mucosally primed to either H7 flagellin or other *E. coli* flagellins, which share highly conserved regions at the N and C termini (42), prior to immunization, as it has been shown that prior mucosal priming is required to generate an intestinal antigen-specific IgA response to systemic immunization with either trinitrophenyl-keyhole limpet hemocyanin or inactivated polio vaccine in mice and humans, respectively (18, 21). A second possibility is that the mucosal IgA response induced by systemically administered H7 represents an inherent capability of H7 to direct immune responses toward the mucosal surfaces.

Finally, further intriguing findings in this study relate to the dynamics of the antibody response generated by i.m. immunization of H7. First, peak IgA levels occurred after the second immunization in both serum and nasal secretions, whereas H7-specific IgG continued to rise after the third immunization. One possible explanation is that flagellin administered on the third immunization was excluded or blocked by preexisting antibodies and therefore was not available to stimulate further antibody production. The subsequent fall in H7-specific IgA, but not IgG, could be explained by differences in their respective half-lives: serum half-lives of IgA and IgG in calves have been estimated to be approximately 2.5 days for IgA and 16 days for IgG (5). Therefore, if the third immunization failed to induce any further antibody production, H7-specific IgA would fall whereas IgG levels would be maintained for a longer period.

Secondly, whereas oral bacterial challenge with *E*. coli O157:H7 resulted in an increase in H7-specific IgA antibodies in mucosal and serum samples from unvaccinated and mucosally immunized calves, no such increase in anti-H7 IgA occurred in the i.m. immunized group. However, as a significant positive correlation existed between levels of anti-H7 and anti-O157 LPS IgA in postchallenge rectal-swab samples from both systemically immunized and nonvaccinated calves, it is likely that the lack of an obvious increase in anti-H7 IgA in the i.m. immunized group was, at the rectum at least, due to lower levels of bacterial colonization. The lack of a correlation between levels of H7 and LPS IgA in postchallenge serum and nasal secretions from i.m. immunized calves could be explained by the existence of high levels of vaccine-induced anti-H7 IgA antibodies in these samples prior to oral challenge.

In conclusion, we have shown that systemic, but not rectal, immunization of cattle with H7 flagellin resulted in reduced colonization rates and delayed peak shedding of *E. coli* O157:H7 following experimental challenge but did not affect total bacterial fecal shedding. This effect was associated with a widespread systemic and mucosal antibody response. Furthermore, both anti-IgA and -IgG antibodies were shown to inhibit binding of *E. coli* O157:H7 to the intestinal epithelium in vitro. These results indicate that H7 flagellin may be a useful component of a systemic vaccination against *E. coli* O157:H7 in cattle.

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